Vibrio cholerae residing in food vacuoles expelled by protozoa are more infectious *in vivo*

Gustavo Espinoza-Vergara[1,2], Parisa Noorian[1,2], Cecilia A. Silva-Valenzuela[3], Benjamin B. A. Raymond[2], Christopher Allen[4], M. Mozammel Hoque[2], Shuyang Sun[2], Michael S. Johnson[4], Mathieu Pernice[5], Staffan Kjelleberg[6,7], Steven P. Djordjevic[2], Maurizio Labbate[4], Andrew Camilli[3], Diane McDougald[2,6*].

[1]School of Biotechnology and Biomolecular Science, University of New South Wales, Sydney, 2052 Australia.

[2] The ithree Institute, University of Technology Sydney, Sydney 2007, Australia.

[3] Department of Molecular Biology and Microbiology, Howard Hughes Medical Institute, Tufts University, School of Medicine, Boston, MA 02111, USA.

[4]School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney 2007, Australia.

[5]Climate Change Cluster, University of Technology Sydney, Sydney 2007, Australia

[6]Singapore Centre for Environmental Life Sciences Engineering, School of Biological Sciences, Nanyang Technological University, Singapore 637551.

[7]School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, 2052 Australia.

*Correspondence to: diane.mcdougald@uts.edu.au

ABSTRACT

Vibrio cholerae interacts with many organisms in the environment, including heterotrophic protists (protozoa). Several species of protozoa have been reported to release undigested bacteria in expelled food vacuoles (EFVs) when feeding on some pathogens. While the production of EFVs has been reported, their biological role as a vector for the transmission of pathogens remains unknown. Here we report that ciliated protozoa release EFVs containing *V. cholerae*. The EFVs are stable, the cells within are protected from multiple stresses and vast numbers escape when incubated at 37°C or in the presence of nutrients. We show that OmpU, a major outer membrane protein positively regulated by ToxR, plays a role in the production of EFVs. Importantly, cells released from EFVs have growth and colonisation advantages over planktonic cells both *in vitro* and *in vivo*. Our results suggest that EFVs facilitate *V. cholerae* survival in the environment, enhancing infectious potential and may significantly contribute to the dissemination of epidemic *V. cholerae* strains. These results establish an improved understanding of the mechanisms of persistence and the modes of transmission of *V. cholerae* and may further apply to other opportunistic pathogens that have been shown to be released by protists in EFVs.

INTRODUCTION

Vibrio cholerae is an aquatic bacterium that is the aetiological agent of the acute diarrhoeal disease cholera, which is endemic in many countries. Outbreaks are linked to inadequate access to clean water and sanitation and it is estimated that there are 1.3 to 4.0 million cases and 21,000 to 143,000 deaths annually worldwide[1]. Reports have shown that both toxigenic (produces cholera toxin (CT)) and non-toxigenic (CT negative) *V. cholerae* are globally-distributed aquatic bacteria. Despite strong evidence that the primary habitat of *V. cholerae* is the marine environment[2] (e.g. estuarine and coastal waters[3]), there are also reports showing the persistence of *V. cholerae* in fresh water systems[4]. In the aquatic environment, *V. cholerae* interacts with many organisms, including protozoa, aquatic plants, phytoplankton, zooplankton and sediments, that may all act as reservoirs. These natural reservoirs may play a critical role in survival of *V. cholerae* in inter-epidemic periods and may also be responsible for the development of virulence[5]. For example, it has been shown that *V. cholerae* colonises and reproduces in copepods, and copepod blooms might result in numbers of *V. cholerae* required for an infective dose[6]. Furthermore, cholera outbreaks have been linked to ingestion of fresh fish[7], shellfish, crabs and oysters[8].

Protozoa take up bacterial prey into phagosomes that become acidified and filled with enzymes resulting in digestion. However, several species of ciliates and amoebae can package and release undigested cells when feeding on certain species of bacterial pathogens. For example, the amoebae *Acanthamoeba* spp. and *Dictyostelium discoideum* and ciliates such as *Tetrahymena* spp., *Colpodia* spp. and *Glaucom* spp. release food vacuoles containing live bacterial cells when feeding on *Salmonella enterica*, *Legionella pneumophila*, *Mycobacterium smegmatis*, *Listeria monocytogenes, Campylobacter jejuni, Pseudomonas aeruginosa* and *Helicobacter pylori*[9-17]. Importantly, bacteria inside EFVs are more resistant to acidic environments[18], freeze/thaw, sonication and 24 h exposure to cooling tower

biocides[19]. Cells within EFVs show enhanced survival under starvation conditions and may remain viable for at least six months[10]. Such resistance to stress and long-term starvation may facilitate the subsequent uptake by and infection of a host[11], although this has not yet been demonstrated. This represents a major gap in our understanding of epidemiology of many infectious diseases.

V. cholerae expresses defences against *Tetrahymena pyriformis*, including the PrtV protease[20], chitin-induced production of ammonia[21] and production of the pigment pyomelanin[22]. However, the production of *V. cholerae*-EFVs has not been previously demonstrated. Here we report that co-incubation of *V. cholerae* with different ciliates results in the release of *V. cholerae* into EFVs to the extracellular environment. We also demonstrate that *V. cholerae*-EFVs survive better than planktonic free-living cells under different stresses and show an increased infectious potential. Taken together, our results suggest that *V. cholerae*-EFVs lead to increased survival of *V. cholerae* epidemics strains in both the natural and the host environment, significantly contributing to the dissemination and infection of *V. cholerae*.

RESULTS

The production of *V. cholerae*-EFVs by *T. pyriformis* is dependent on prey cell number and enhanced by bacterial protein synthesis. This study shows that when *V. cholerae* and *T. pyriformis* are co-incubated, EFVs containing live undigested bacteria are released into the environment through the ciliate cytoproct (anus) (Fig. 1a, Supplementary Fig. 1 and Supplementary Video 1). Transmission electron microscopy (TEM) of the EFVs reveal that *V. cholerae* is packaged into multilamellar vacuoles, similar to what has been reported for EFVs containing *L. pneumophila*[9,23] (Fig. 1b, Supplementary Fig. 2a). The production of *V. cholerae*-EFVs increases as the bacterial concentration increases, illustrating that EFV production is dependent on prey cell number (Fig. 1c). EFVs are also produced when *V. cholerae* is co-incubated with *Acanthamoeba castellanii* (Supplementary Fig. 2b).

To determine if active *V. cholerae* is necessary for EFV production, bacteria were pre-treated with sub-lethal concentrations of gentamicin to inhibit protein synthesis. Inhibition of protein synthesis resulted in a significant decrease in production of EFVs by *T. pyriformis* (Fig. 1d and Supplementary Fig. 3a). Furthermore, there is a significant reduction in EFV production with heat-killed *V. cholerae* (Supplementary Fig. 3b). These results indicate that specific factor(s) are actively produced by *V. cholerae* when inside the protozoan phagosome, contributing to release of EFVs.

Co-incubation of different *V. cholerae* strains with several ciliated protozoa also results in the production of EFVs. To further determine if the production of EFVs is a general response, different *V. cholerae* and ciliate wild type strains were tested for EFV production. Results showed that the co-incubation of *V. cholerae* O1 N16961, A1552, C6706 and HC1037 with *T. pyriformis*, *Tetrahymena malaccensis*, *Tetrahymena* sp., *Uronema marinum* and *Tetrahymena thermophila* led to the release of *V. cholerae*-EFVs to the extracellular space (Fig. 2 a-e). Higher number of EFVs were observed in the co-incubations with *T.*

106 *thermophila* at 30°C (Fig. 2e) suggesting that increased temperature might enhance EFV production. These results show that predation of *V. cholerae* by ciliated protozoa at both RT
108 and 30°C results in the production of EFVs. We also tested different *Vibrio* spp. (Fig. 2f) showing that there are differences in EFV production depending on strains.

110 **The *V. cholerae* outer membrane protein OmpU is involved in the release of EFVs**. In order to identify the potential factor(s) responsible for the production of EFVs, various *V.*
112 *cholerae* A1552 mutants with deletions in genes related to grazing resistance/biofilm formation[22,24,25], transcriptional regulation[26-29], motility[30], acid resistance[31], outer membrane
114 proteins[32,33], aminoacyl lipid modification[34], types I[35], II[36-38] and VI[39] secretion system and intracellular survival and multiplication were tested[40]. No growth defect in LB was observed
116 for any of the mutants used in this study. Before each co-incubation, bacteria were adjusted to OD_{600}: 1.00-1.04 (approximately 10^9 CFU ml^{-1}) in 0.55 × NSS and serially diluted to the
118 desired concentration. Compared to the wild type, a significant decrease in the number of EFVs were observed when *toxR* and *ompU* mutants were used as prey (Table 1).

120 ToxR is the transcriptional regulator of *ompU.* Thus, to determine if the defect in EFV production in the Δ*toxR* strain is due to loss of *ompU* expression, or if other genes in the
122 virulence operon regulated by ToxR are involved, both Δ*ompU* and Δ*toxR* strains were complemented with *ompU.* In addition, as the operon that encodes *ompU* includes *dacB*
124 (carboxypeptidase located downstream of *ompU*), a *dacB* deletion mutant was also constructed and tested. Results show that deletion of *dacB* does not affect EFV production,
126 however, complementation of the *ompU* gene in both Δ*ompU* and Δ*toxR* strains restores the number of EFVs back to wild type levels (Fig. 3). These results indicate that OmpU, an outer
128 membrane protein involved in the resistance to antimicrobial peptides[41], bile salts[42] and organic acids[43] and positively regulated by the master regulator of virulence, ToxR, plays an
130 important role in the production of EFVs.

EFVs protect cells from stress (acid stress, antimicrobials and starvation). Bacterial cells inside the EFVs are potentially protected from various environmental and host stresses. Therefore, *V. cholerae*-EFVs were purified by filtration, washed and exposed to pH stress (pH = 3.4, the pH of the human stomach[44]) along with planktonic *V. cholerae* cells as controls. The viability of cells within the EFVs was only slightly affected (< 1 log reduction) whereas planktonic *V. cholerae* were completely killed after 40 min of incubation (Fig. 4a). Thus, EFVs can protect *V. cholerae* from low pH conditions that would be encountered upon entering a human host gut. Another common stress encountered by bacteria is exposure to biocides; thus, the experiment was repeated using gentamicin at a bactericidal concentration (300 μg ml^{-1}) at room temperature (RT). While planktonic *V. cholerae* cells were again completely eradicated, the cells within EFVs showed no loss of viability (Fig. 4b). Therefore, our data show that EFVs act as a protective barrier against different *V. cholerae* stressors.

Starvation is a common environmental stress for bacteria in aquatic environments[45]. Many marine bacteria can survive long periods under starvation conditions, while others decline in number over time. To determine whether cells within the EFVs can survive long-term starvation, EFVs were collected, re-suspended in artificial seawater (0.55 × NSS) and stored at RT. Viability was assessed and compared to planktonic *V. cholerae* maintained under the same conditions. After one week, there was an approximate 2.5 log decrease in the viability of the planktonic cells (Fig. 4c). In contrast, the cells within the EFVs maintained viability for at least 3 months (< 0.5 log reduction). This result confirms that EFVs confer a fitness advantage to *V. cholerae* and increases viability in seawater, thus, contributing to their persistence in the environment.

The escape of *V. cholerae* from EFVs is mediated by temperature and the presence of nutrients. For EFVs to be an ecologically relevant mechanism of protection and transmission for pathogens in the environment, the cells within must be able to escape and propagate.

156 EFVs that were incubated in LB broth at 37°C escape very quickly (15 – 30 min) and begin dividing (Fig. 5a and Supplementary Video 4 and 5). At 37°C in 0.55 × NSS without carbon
158 or nutrient sources, the cells in the centre of the EFVs can be seen to increase motility and within 4 h escaped the EFVs, but at a slower rate (Fig. 5b). The experiment was repeated
160 with EFVs that had been stored in 0.55 × NSS at RT (~22°C) for 2 months. Cell escape and propagation from the EFVs was observed in LB broth within 3 h of incubation (Fig. 5c), but
162 no EFV escape was observed during the preceding 2 months (Fig. 5d). Thus, the escape of *V. cholerae* from EFVs is triggered by increased temperature and the presence of nutrients.

164 **Cells in EFVs show an *in vitro* fitness advantage**. We next tested the fitness of *V. cholerae* cells contained in EFVs and of planktonic cells for growth in nutrient media (LB). The *V.*
166 *cholerae* A1552 wild type strain was used to produce 24 h old EFVs and competed against a Δ*lacZ* isogenic strain that had been grown *in vitro* and acclimatised in 0.55 × NSS before
168 inoculation. The *in vitro* competition was performed by inoculating 50 µl of a 0.55 × NSS suspension containing purified EFVs (approximately 6 x 10^4 EFVs ml^{-1}) and Δ*lacZ* isogenic
170 strain planktonic cells (approximately 6 x 10^5 cells ml^{-1}; to differentiate planktonic cells from cells originating from EFVs by growth in the presence of X-gal for blue/white screening) in
172 LB broth and incubating at 37ºC overnight with agitation. The competitive index (CI) was calculated as CFU of EFVs/CFU of Δ*lacZ* wild-type corrected by the number of viable *V.*
174 *cholerae* cells in EFVs (Supplementary Fig. 5a-c and Supplementary File 1 and 2). As a result, the *in vitro* CI (Fig. 6a, median 6.5) suggests that the EFVs confer a growth advantage
176 for *V. cholerae* when nutrients are encountered.

Purified *V. cholerae*-EFVs are primed for infection *in vivo*. Since EFVs are produced in
178 large numbers under intense predation, and cells within the EFVs are protected against a range of stresses and can maintain viability long-term under environmental conditions, it
180 follows that these EFVs may be infective when consumed by a host. In order to assess the

infectivity of *V. cholerae*-EFVs, an infant mouse model of colonisation was employed. For this, 50 µl of the same inoculum used for *in vitro* competition was used to infect the animals (described in Methods). After 24 h of infection, the CI was calculated from cells obtained from the small intestine of each animal. Despite considerable variability in the results, *V. cholerae*-EFVs outcompeted the *in vitro* grown bacteria *in vivo*, with median CI significantly higher than 1.0 ($P<0.0001$, Wilcoxon Signed Rank Test). The *in vivo* CI (Fig. 6b, median 14.7) demonstrates that *V. cholerae*-EFVs have a significant colonisation advantage compared to planktonic cells.

***V. cholerae*-EFVs maintain the *in vivo* hyperinfectivity for 6 weeks**. The incubation of *V. cholerae* within EFVs in the environment might result in long-time periods before they are ingested by a host. In order to test if aged EFVs maintain the hyperinfective phenotype, purified EFVs were incubated in 0.55 × NSS for 6 weeks at RT and used for *in vitro* and *in vivo* competition assays as described previously. Contrary to previous results, the 6-week-old EFVs showed an *in vitro* growth disadvantage (median 0.07) compared to the control (planktonic 6-week-old *V. cholerae*, median 1.43) (Fig. 6c). However, the presence of many aggregates were detected after the overnight growth in LB broth, suggesting that *V. cholerae*-EFVs did grow as aggregated bacteria, a fact that could affect the calculation of CFUs of the escaped *V. cholerae*. In contrast, the 6-week-old EFVs still showed a colonisation advantage (median 1.74) compared to the control (median 0.56) (Fig. 6d), confirming that long-term incubation did not affect the hyperinfective capability of *V. cholerae*-EFVs.

***V. cholerae*-EFVs are not degraded at 37ºC and low pH but are digested in the presence of deoxycholic acid.** In order to assess whether the EFVs might be degraded either in the stomach or the small intestine, EFVs were incubated in two conditions. First, purified EFVs were re-suspended in 0.55 × NSS at pH 3.4 and incubated at 37ºC for 4 h. Imaging results

showed no escape of *V. cholerae* from EFVs (Fig. 6e, left panel). However, exposure of the EFVs to 0.4 % of deoxycholic acid resulted in immediate digestion of EFVs (Fig. 6e, right panel). Together these results suggest that *V. cholerae* would remain inside of EFVs when transiting through the stomach, but would be released from the EFVs at the site of colonisation (small intestine) in the presence of bile.

DISCUSSION

Results here suggest that when numbers of *V. cholerae* are high in the environment, e.g. during disease outbreaks, there would be intense predation pressure and some of these protists release EFVs into the water column (Supplementary Fig. 6). Although the production of EFVs has been shown for other pathogens, it has not been demonstrated whether this process is mediated by the protist or bacteria. Here we show that OmpU plays a key role in the production of EFVs by *V. cholerae*, demonstrating that bacterial factors positively contribute to this process. After ingestion by *T. pyriformis*, *V. cholerae* in phagosomes encounters the presence of an adverse environment characterised by the presence of low pH and cationic antimicrobial peptides[46,47]. As it has been shown previously in *V. cholerae*, OmpU enables resistance to such environments. For example, reports have shown that OmpU protects *V. cholerae* from antimicrobial peptides[41,48,49], low pH[43] and bile[50]. In addition, it has been shown that in *V. cholerae* OmpU is involved in intestinal colonisation[32] and, in other *Vibrio* spp., OmpU is essential for invasion and infection of oysters[48,51]. As a result, the egestion of *V. cholerae* from EFVs is promoted by an outer membrane protein that is essential for the pathogenesis of this bacterium.

The fact that OmpU protects *V. cholerae* cells from these types of stresses indicates that once inside the phagosome, OmpU probably acts to resist digestion of the bacterial cells. This will result in a high number of undigested cells within the vacuole. The undigested cells

remaining in the phagosome may trigger the expulsion of vacuoles containing bacteria from *T. pyriformis* as previously demonstrated[52].

Since EFVs confer an advantage to *V. cholerae* for survival under stressful conditions, the EFV cells are protected from various environmental stresses and pH stress that would be encountered upon ingestion. The EFVs would enhance survival of cells passaging through the stomach and as the EFVs contain numerous cells, would increase numbers of *V. cholerae* that reach the small intestine (Fig. 4). Our mouse colonisation data shows that *V. cholerae* in EFVs can outcompete planktonic cells, suggesting that EFVs might protect cells and may also enhance efficient infection, possibly through improved survival upon exposure to gastric acid and increased resistance to host antimicrobial defences through active expression of *ompU*. Furthermore, as stated above, OmpU is critical for intestinal colonisation[32], suggesting that the expression of OmpU in EFVs might be responsible for the *in vivo* colonisation advantage. We suggest that the findings reported here establish a novel understanding of the mechanisms of persistence and the modes of transmission of *V. cholerae* and may further apply to other opportunistic pathogens that have been shown to be released by protists in EFVs. Hence, protozoan EFVs may constitute a mechanism for transmission and infection more broadly as has been previously speculated[4,19].

METHODS

Strains and growth conditions. Organisms used in this study are listed in Supplementary Table 1. Bacterial strains were routinely grown in lysogeny broth (LB) and on LB agar plates. *V. cholerae* mutants were constructed by splicing by overlap extension PCR[53] and natural transformation[54]. Complementation was done using the expression vector pBAD24. Bacteria carrying the vector were grown in LB broth at 37ºC containing ampicillin 100 µg ml^{-1} and, 0.2% arabinose for gene expression. Environmental isolates of

Vibrio spp. were routinely grown in LB broth and LB agar plates supplemented with 2% NaCl and incubated at 28°C.

Tetrahymena spp. were routinely passaged in 15 ml growth medium containing peptone-yeast-glucose (PYG) (20 g l^{-1} proteose peptone, 1 g l^{-1} yeast extract) supplemented with 1 l 0.1 × M9 minimal medium (6 g l^{-1} NaH_2PO_4, 3 g l^{-1} K_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl) and 0.1 M sterile-filtered glucose in 25 cm^2 tissue culture flasks with ventilated caps (Sarstedt Inc., Nümbrecht, Germany) and incubated statically at room temperature (RT). *U. marinum* was routinely grown in 0.55 × NSS medium (8.8 g l^{-1} NaCl, 0.735 g l^{-1} Na_2SO_4, 0.04 g l^{-1} $NaHCO_3$, 0.125 g l^{-1} KCl, 0.02 g l^{-1} KBr, 0.935 g l^{-1} $MgCl_2.6H_2O$, 0.205 g l^{-1} $CaCl_2.2H_2O$, 0.004 g l^{-1} $SrCl_2.6H_2O$ and 0.004 g l^{-1} H_3BO_3) supplemented with 1% heat-killed *Pseudomonas aeruginosa* PAO1 (HKB) in a 25 cm^2 tissue culture flask, and further incubated at RT statically for 2 days before enumeration and use.

Prior to experiments, 500 µl of *Tetrahymena* spp. were passaged in 20 ml of 0.55 × NSS medium supplemented with 1% heat- killed *P. aeruginosa* PAO1 (HKB) in a 25 cm^2 tissue culture flask, and further incubated at RT statically for 2 days before enumeration and use. This process is necessary to remove the nutrient media and to acclimatise the ciliate to phagotrophic feeding.

To prepare heat-killed bacteria (HKB), *P. aeruginosa* and *V. cholerae* were grown overnight in LB at 37°C with shaking at 200 rpm and adjusted to (OD_{600}=1.0; 10^9 cells ml^{-1}) in 0.55 × NSS. The tubes were then transferred to a water bath at 65°C for 2 h, and then tested for viability by plating on LB agar plates at 37°C for 2 days. HKB stocks were stored at -20°C.

Production of EFVs containing *V. cholerae*. To produce EFVs, *V. cholerae* A1552 was co-incubated with *T. pyriformis* in 0.55 × NSS. Briefly, *T. pyriformis* were enumerated by microscopy and adjusted to 10^3 cells ml^{-1} and added to co-cultures of *V. cholerae* A1552

adjusted to 10^8 cells ml^{-1} in 0.55 × NSS using a spectrophotometer (OD_{600} nm). After overnight incubation at RT, samples were analysed using an inverted epifluorescence microscope (Nikon Eclipse Ti inverted microscope) to detect the presence of EFVs in the supernatant. To purify *V. cholerae*-EFVs, supernatants were filtered (by gravity) several times through 8 µm filters (Millipore) and the filters containing EFVs suspended in 1 ml 0.55 × NSS. The EFVs were incubated for 1 h with gentamicin 300 µg ml^{-1} at RT to kill any remaining extracellular bacteria. After gentamicin treatment, *V. cholerae*-EFVs pellets were collected by centrifugation (3220 × *g* for 20 min), washed three times in 0.55 × NSS and suspended in 1 ml of 0.55 × NSS. Finally, the number of *V. cholerae*-EFVs was determined by microscopy after 48 h of co-incubation (time needed for the eradication of all extracellular bacteria).

Enumeration of live/dead *V. cholerae* in EFVs. In order to establish the number of viable *V. cholerae* in EFVs, a genomic staining assay was conducted. Briefly, EFVs were produced and collected as above and suspended in 1 ml of 0.55 × NSS. The EFVs were stained with LIVE/DEAD ™ BacLight ™ Bacterial Viability Kit for microscopy (Invitrogen) following manufacturer's instructions. After staining, the sample was centrifuged (7607 × g, 5 min) to remove the staining solution and resuspended in 1 ml of 0.55 × NSS. Eight µl of sample were placed on a glass slide, covered with a coverslip (1.5 mm thickness) and sealed with nail polish. Stained EFVs were immediately analysed by confocal microscopy (Nikon A1 confocal laser scanning microscope) to assess the number of live (green) and dead (red) bacterial cells.

Survival of *V. cholerae*-EFVs under stress conditions. To assess the effect of stress conditions on the viability of *V. cholerae* within EFVs, two treatments were performed independently. For the acid tolerance experiments, *V. cholerae*-EFVs were obtained as described above and suspended in either 0.55 × NSS or NSS adjusted to pH 3.4 (with 1N

HCl). Incubation of the *V. cholerae*-EFVs were carried out in triplicate for 60 min in a 96 well plate at RT with agitation (60 rpm). The numbers of viable bacteria were determined at different time points by adding 1% of Triton-X100 (Sigma) to each well at 0, 20, 40 and 60 min (to release the *V. cholerae* cells from the EFVs, Supplementary Fig. 4a-c) and plating serial dilutions on LB plates. For the gentamicin assay, *V. cholerae*-EFVs were exposed to 300 µg ml^{-1} in 0.55 × NSS at RT and 60 rpm in a 96 well plate. After 1 h incubation, 1% Triton-X100 (Sigma) was added to each well and serial dilutions were plated on LB. As a control, planktonic *V. cholerae* adjusted to ~10^6 cells ml^{-1} in 0.55 × NSS was used for each of the three conditions.

Escape of *V. cholerae* from EFVs. To obtain images and videos of *V. cholerae* cells escaping from EFVs, the EFVs were collected as described above, suspended in LB broth or 0.55 × NSS and 1 ml of the suspension added to a 24-well glass bottom microtiter plate. Plates were incubated at 37ºC or RT in a confocal microscope (Nikon A1 confocal laser scanning microscope) and videos or pictures were taken.

Incubation of EFVs at low pH and in the presence of deoxycholic acid. Purified *V. cholerae*-EFVs were incubated at 37°C for 4 h in 0.55 × NSS at pH 3.4. To test the effect of deoxycholic acid (component of bile) on the EFVs, treatments with 0.4% deoxycholic acid were performed at 37°C after 4 h of incubation in 0.55 × NSS at pH 3.4

Infant mouse colonisation experiments. Five-day-old litters of CD1 mice were inoculated orogastrically as described[55] with 50 µl of inoculum containing ~10^6 Rifampicin-resistant *V. cholerae* A1552 in EFVs (24 h old) and ~10^6 CFU of an isogenic competing strain, *V. cholerae* A1552 Δ*lacZ*, which was prepared by growth *in vitro* to stationary phase in LB broth at 37°C with aeration. In parallel, 2 µl of inoculum was diluted into 2 ml of LB broth in culture tubes and competed *in vitro* for 18 h with aeration at 37°C. After 24 h, mice were

326 euthanised, the small intestine was removed and homogenised in 1 ml of LB broth supplemented with 20% glycerol.

328 For the 6 weeks-old EFVs experiment, *in vitro* growth and *in vivo* infections were performed as described above. As a control, 6 weeks-starved planktonic *V. cholerae* in 0.55 × NSS at
330 RT was used. The ratios of wild type to Δ*lacZ V. cholerae* in the input (inoculum) and outputs were determined by plating serial dilutions on LB agar supplemented with rifampicin
332 100 µg ml^{-1} and X-Gal 80 µg ml^{-1}. The CI was calculated as the output ratio divided by the input ratio corrected by the number of *V. cholerae* into EFVs.

334 All animal procedures were conducted in accordance with the rules of the Department of Laboratory Animal Medicine at Tufts University School of Medicine. Five-day-old CD-1
336 infant mice (both male and female animals) were used for the infection experiments to obtain an accurate median for statistical analyses. All animals were obtained from Charles River
338 Laboratories.

Transmission electron microscopy. Cell cultures were fixed for 24 h at 4˚C by immersion in
340 a fixative solution containing 3% glutaraldehyde in PBS buffer (0.1 M phosphate, pH 7.5) and then stored in PBS buffer (0.1 M, pH 7.5) at 4˚C until further processing. Samples were
342 subsequently post-fixed for 1 h in a solution containing 1% osmium tetroxide in PBS (1X, final pH 7.5), washed with MilliQ water and dehydrated in an increasing gradient of ethanol before
344 infiltration and embedding in SPURR resin. Resin blocs were then cut into 90 nm sections using an Ultracut UC6 microtome (Leica Microsystems, Australia). Selected sections
346 containing cells and EFVs were stained on finder grids (Electron Microscopy Sciences, Hatfield, PA, USA) with uranyl acetate and lead citrate. Stained sections on finder grids were
348 viewed at 200 kV accelerating voltage using a FEI Tecnai G2 20 Transmission Electron

Microscope within the Mark Wainwright Analytical Centre: Electron Microscope Unit (University of New South Wales).

Data analysis. Statistical analysis was performed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com). Data that did not follow Gaussian distribution was determined by analysing the frequency distribution graphs and was transformed using natural log. Two-tailed student's t-tests were used to compare means between experimental samples and controls. For experiments including multiple samples, one-way ANOVAs or 2-way ANOVAs were used for the analysis and Dunnett's Multiple Comparison Test provided the post-hoc comparisons of means. For the mice colonisation experiments, the data was analysed by using a non-parametrical test for medians that follow Gaussian distribution (Wilcoxon Signed Rank Test or Mann-Whitney Test) for a non-normally distributed data.

Data Availability. The data that support the findings of this study are available from the corresponding author upon request.

References

1 Ali, M., Nelson, A. R., Lopez, A. L. & Sack, D. A. Updated global burden of cholera in endemic countries. *PLoS Negl Trop Dis* **9**, e0003832, doi:10.1371/journal.pntd.0003832 (2015).

2 Colwell, R. & Huq, A. Marine ecosystems and cholera. *Hydrobiologia* **460**, 141-145, doi:10.1023/A:1013111016642 (2001).

3 Martinelli Filho, J. E., Lopes, R. M., Rivera, I. N. G. & Colwell, R. R. *Vibrio cholerae* O1 detection in estuarine and coastal zooplankton. *J Plankton Res* **33**, 51-62, doi:10.1093/plankt/fbq093 (2010).

4 Nair, G. B. *et al.* Ecology of *Vibrio cholerae* in the freshwater environs of Calcutta, India. *Microbial Ecol* **15**, 203-215 (1988).

5 Vezzulli, L., Pruzzo, C., Huq, A. & Colwell, R. R. Environmental reservoirs of *Vibrio cholerae* and their role in cholera. *Environ Microbiol Rep* **2**, 27-33, doi:10.1111/j.1758-2229.2009.00128.x (2010).

6 Colwell, R. R. *et al.* Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci U S A* **100**, 1051-1055, doi:10.1073/pnas.0237386100 (2003).

7 Acosta, C. J. *et al.* Cholera outbreak in southern Tanzania: risk factors and patterns of transmission. *Emerg Infect Dis* **7**, 583-587, doi:10.3201/eid0707.010741 (2001).

8 Rabbani, G. H. & Greenough, W. B., 3rd. Food as a vehicle of transmission of cholera. *J Diarrhoeal Dis Res* **17**, 1-9 (1999).

9 Berk, S. G. *et al.* Packaging of live *Legionella pneumophila* into pellets expelled by *Tetrahymena* spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. *J Appl Environ Microbiol* **74**, 2187-2199, doi:10.1128/AEM.01214-07 (2008).

10 Bouyer, S., Imbert, C., Rodier, M.-H. & Héchard, Y. Long-term survival of *Legionella pneumophila* associated with *Acanthamoeba castellanii* vesicles. *Environ Microbiol* **9**, 1341-1344, doi:10.1111/j.1462-2920.2006.01229.x (2007).

11 Denoncourt, A. M., Paquet, V. E. & Charette, S. J. Potential role of bacteria packaging by protozoa in the persistence and transmission of pathogenic bacteria. *Front Microbiol* **5**, 240, doi:10.3389/fmicb.2014.00240 (2014).

12 Gourabathini, P., Brandl, M. T., Redding, K. S., Gunderson, J. H. & Berk, S. G. Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl Environ Microbiol* **74**, 2518 (2008).

13 Koubar, M., Rodier, M.-H., Garduño, R. A. & Frère, J. Passage through *Tetrahymena tropicalis* enhances the resistance to stress and the infectivity of *Legionella pneumophila*. *FEMS Microbiol Lett* **325**, 10-15, doi:10.1111/j.1574-6968.2011.02402.x (2011).

14 Marciano-Cabral, F. & Cabral, G. *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev* **16**, 273-307, doi:10.1128/CMR.16.2.273-307.2003 (2003).

15 Paquet, V. E. & Charette, S. J. Amoeba-resisting bacteria found in multilamellar bodies secreted by *Dictyostelium discoideum*: social amoebae can also package bacteria. *FEMS Microbiol Ecol* **92**, fiw025, doi:10.1093/femsec/fiw025 (2016).

16 Raghu Nadhanan, R. & Thomas, C. J. *Colpoda* secrete viable *Listeria monocytogenes* within faecal pellets. *Environ Microbiol* **16**, 396-404, doi:10.1111/1462-2920.12230 (2013).

17 Trigui, H., Paquet, V. E., Charette, S. J. & Faucher, S. P. Packaging of *Campylobacter jejuni* into multilamellar bodies by the ciliate *Tetrahymena pyriformis*. *J Appl Environ Microbiol* **82**, 2783-2790, doi:10.1128/AEM.03921-15 (2016).

18 Rehfuss, M. Y. M., Parker, C. T. & Brandl, M. T. *Salmonella* transcriptional signature in *Tetrahymena* phagosomes and role of acid tolerance in passage through the protist. *ISME J* **5**, 262-273, doi:10.1038/ismej.2010.128 (2011).
19 Berk, S. G., Ting, R. S., Turner, G. W. & Ashburn, R. J. Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. *Appl Environ Microbiol* **64**, 279-286 (1998).
20 Vaitkevicius, K. *et al.* A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *Proc Natl Acad Sci, U.S.A* **103**, 9280-9285, doi:10.1073/pnas.0601754103 (2006).
21 Sun, S., Tay, Q. X. M., Kjellberg, S., Rice, S. A. & McDougald, D. Quorum sensing-regulated chitin metabolism provides grazing resistance to *Vibrio cholerae* biofilms. *ISME J* **9**, 1812-1820 (2015).
22 Noorian, P. *et al.* Pyomelanin produced by *Vibrio cholerae* confers resistance to predation by *Acanthamoeba castellanii*. *FEMS Microbiol Ecol* **93**, fix147, doi:10.1093/femsec/fix147 (2017).
23 Berk, S. G., Ting, R. S., Turner, G. W. & Ashburn, R. J. Production of respirable vesicles containing live Legionella pneumophila cells by two Acanthamoeba spp. *Appl Environ Microbiol* **64**, 279-286 (1998).
24 Sun, S., Kjelleberg, S. & McDougald, D. Relative contributions of *Vibrio* polysaccharide and quorum sensing to the resistance of *Vibrio cholerae* to predation by heterotrophic protists. *PLoS One* **8**, e56338-e56338, doi:10.1371/journal.pone.0056338 (2013).
25 Casper-Lindley, C. & Yildiz, F. H. VpsT is a transcriptional regulator required for expression of vps biosynthesis genes and the development of rugose colonial morphology in *Vibrio cholerae* O1 El Tor. *J Bacteriol* **186**, 1574-1578, doi:10.1128/JB.186.5.1574-1578.2004 (2004).
26 Jobling, M. G. & Holmes, R. K. Characterization of hapR, a positive regulator of the *Vibrio cholerae* HA/protease gene hap, and its identification as a functional homologue of the *Vibrio harveyi* luxR gene. *Mol Microbiol* **26**, 1023-1034 (1997).
27 Pratt, J. T., McDonough, E. & Camilli, A. PhoB regulates motility, biofilms, and cyclic di-GMP in *Vibrio cholerae*. *J Bacteriol* **191**, 6632, doi:10.1128/JB.00708-09 (2009).
28 Miller, V. L. & Mekalanos, J. J. Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. *Proc Natl Acad Sci U S A* **81**, 3471-3475, doi:10.1073/pnas.81.11.3471 (1984).
29 Chourashi, R. *et al.* Role of a sensor histidine kinase ChiS of *Vibrio cholerae* in pathogenesis. *Int J Med Microbiol* **306**, 657-665, doi:10.1016/j.ijmm.2016.09.003 (2016).
30 Klose, K. E. & Mekalanos, J. J. Differential regulation of multiple flagellins in Vibrio cholerae. *J Bacteriol* **180**, 303-316 (1998).
31 Merrell, D. S. & Camilli, A. Regulation of *Vibrio cholerae* genes required for acid tolerance by a member of the "ToxR-like" family of transcriptional regulators. *J Bacteriol* **182**, 5342-5350, doi:10.1128/jb.182.19.5342-5350.2000 (2000).
32 Sperandio, V., Girón, J. A., Silveira, W. D. & Kaper, J. B. The OmpU outer membrane protein, a potential adherence factor of *Vibrio cholerae*. *Infect Immun* **63**, 4433-4438 (1995).
33 Pohlner, J., Meyer, T. F., Jalajakumari, M. B. & Manning, P. A. Nucleotide sequence of ompV, the gene for a major *Vibrio cholerae* outer membrane protein. *Mol Gen Genet* **205**, 494-500 (1986).
34 Hankins, J. V., Madsen, J. A., Giles, D. K., Brodbelt, J. S. & Trent, M. S. Amino acid addition to *Vibrio cholerae* LPS establishes a link between surface remodeling in Gram-

positive and Gram-negative bacteria. *Proc Natl Acad Sci U S A* **109**, 8722-8727, doi:10.1073/pnas.1201313109 (2012).
35 Lin, W. *et al.* Identification of a vibrio cholerae RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc Natl Acad Sci U S A* **96**, 1071-1076, doi:10.1073/pnas.96.3.1071 (1999).
36 Toma, C. & Honma, Y. Cloning and genetic analysis of the *Vibrio cholerae* aminopeptidase gene. *Infect Immun* **64**, 4495-4500 (1996).
37 Kirn, T. J., Jude, B. A. & Taylor, R. K. A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature* **438**, 863-866, doi:10.1038/nature04249 (2005).
38 Waldor, M. K. & Mekalanos, J. J. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910, doi:10.1126/science.272.5270.1910 (1996).
39 Ishikawa, T., Rompikuntal, P. K., Lindmark, B., Milton, D. L. & Wai, S. N. Quorum sensing regulation of the two hcp alleles in *Vibrio cholerae* O1 strains. *PLoS One* **4**, e6734, doi:10.1371/journal.pone.0006734 (2009).
40 Lomma, M. *et al.* The *Legionella pneumophila* F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cell Microbiol* **12**, 1272-1291, doi:10.1111/j.1462-5822.2010.01467.x (2010).
41 Mathur, J. & Waldor, M. K. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect Immun* **72**, 3577-3583, doi:10.1128/IAI.72.6.3577-3583.2004 (2004).
42 Provenzano, D., Schuhmacher, D. A., Barker, J. L. & Klose, K. E. The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and other pathogenic *Vibrio* species. *Infect Immun* **68**, 1491-1497 (2000).
43 Merrell, D. S., Bailey, C., Kaper, J. B. & Camilli, A. The ToxR-mediated organic acid tolerance response of *Vibrio cholerae* requires OmpU. *J Bacteriol* **183**, 2746-2754, doi:10.1128/JB.183.9.2746-2754.2001 (2001).
44 Russell, T. L. *et al.* Upper gastrointestinal pH in seventy-nine healthy, elderly North American men and women. *Pharm Res* **10**, 187-196 (1993).
45 Östling, J. *et al.* in *Starvation in Bacteria* (ed S. Kjelleberg) 169-174 (Springer, Boston MA, 1993).
46 Jacobs, M. E. *et al.* The *Tetrahymena thermophila* phagosome proteome. *Eukaryot Cell* **5**, 1990-2000, doi:10.1128/EC.00195-06 (2006).
47 Kinchen, J. M. & Ravichandran, K. S. Phagosome maturation: going through the acid test. *Nat Rev Mol Cell Biol* **9**, 781-795, doi:10.1038/nrm2515 (2008).
48 Duperthuy, M. *et al.* The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environ Microbiol* **12**, 951-963, doi:10.1111/j.1462-2920.2009.02138.x (2010).
49 Mathur, J., Davis, B. M. & Waldor, M. K. Antimicrobial peptides activate the *Vibrio cholerae* σE regulon through an OmpU-dependent signalling pathway. *Mol Microbiol* **63**, 848-858, doi:10.1111/j.1365-2958.2006.05544.x (2007).
50 Wibbenmeyer, J. A., Provenzano, D., Landry, C. F., Klose, K. E. & Delcour, A. H. *Vibrio cholerae* OmpU and OmpT porins are differentially affected by bile. *Infect Immun* **70**, 121, doi:10.1128/IAI.70.1.121-126.2002 (2002).
51 Duperthuy, M. *et al.* Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc Natl Acad Sci U S A* **108**, 2993, doi:10.1073/pnas.1015326108 (2011).

52 Thurman, J., Drinkall, J. & Parry, J. D. Digestion of bacteria by the freshwater ciliate *Tetrahymena pyriformis*. *Aquat Microb Ecol* **60**, 163-174, doi:10.3354/ame01413 (2010).
53 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61-68, doi:https://doi.org/10.1016/0378-1119(89)90359-4 (1989).
54 Dalia, A. B., McDonough, E. & Camilli, A. Multiplex genome editing by natural transformation. *Proc Natl Acad Sci U S A* **111**, 8937 (2014).
55 Tischler, A. D. & Camilli, A. Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. *Infect Immun* **73**, 5873-5882, doi:10.1128/IAI.73.9.5873-5882.2005 (2005).
56 Yildiz, F. H. & Schoolnik, G. K. *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc Natl Acad Sci U S A* **96**, 4028 (1999).
57 Lim, B., Beyhan, S., Meir, J. & Yildiz, F. H. Cyclic-diGMP signal transduction systems in *Vibrio cholerae*: modulation of rugosity and biofilm formation. *Mol Microbiol* **60**, 331-348, doi:10.1111/j.1365-2958.2006.05106.x (2006).
58 Valeru, S. P. *et al.* Role of melanin pigment in expression of *Vibrio cholerae* virulence factors. *Infect Immun* **77**, 935-942, doi:10.1128/IAI.00929-08 (2009).
59 Yildiz, F. H., Liu, X. S., Heydorn, A. & Schoolnik, G. K. Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. *Mol Microbiol* **53**, 497-515, doi:10.1111/j.1365-2958.2004.04154.x (2004).

Supplementary Information is available in the online version of the paper.

Acknowledgments: The authors would like to thank Prof Scott A. Rice, Dr Sharon Longford and Dr Branwen Morgan for critical evaluation of the manuscript, Tan Chin Hin Tran for graphic illustrations, Kuan Li for assistance with TEM imaging and Dr Louise Cole for advice with the confocal microscopy. This work was supported by Australian Research Council Discovery Project DP170100453, the United States NIH (AI055058), the Pew Latin American Fellows Program in the Biomedical Sciences from PEW Charitable trusts, the CONICYT Becas Chile doctoral (72140329) and postdoctoral fellowships, the ithree Institute and The Microbial Imaging Facility, Faculty of Science, University of Technology Sydney and financial support from National Research Foundation and Ministry of Education Singapore under its Research Centre of Excellence Program to the Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University.

Author contributions: GEV, PN, AC, DM designed the study and planned the experiments. GEV, PN, CSV, BR, MMH, CA, AC carried out the experiments. GEV, BR and MJ did the microscopy experiments. GEV, PN, and CA carried out the biological assays. GEV, CSV, AC did the infection assay. MP assisted with TEM imaging. GEV, PN, SS, AC, ML, DM contributed to interpretation of the results. GEV and DM took the lead in writing the manuscript. AC, SK, SD, ML and DM provided funding. All authors provided critical feedback and helped shape the manuscript.

Competing interests: The authors declare no competing interests. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to diane.mcdougald@uts.edu.au.

Figure Legends

Figure 1. Production of EFVs containing *V. cholerae*. a, Fixed samples of GFP-tagged (green) *V. cholerae* co-incubated with *T. pyriformis* in 0.55 × NSS. *T. pyriformis* was stained with DAPI (blue nucleus) and FM 4-64 FX was used to stain the EFV membranes (red). The left panel shows intracellular food vacuoles containing GFP-tagged *V. cholerae* (scale bar 15 µm; Supplementary Video 2) and the three right hand panels show an expelled EFV (scale bars 5 µm; Supplementary Video 3). The upper panel shows GFP, the middle stained with FM 4-64 FX and the bottom merged. Images are representative of three independent experiments. **b**, TEM of fixed samples of *T. pyriformis* and *V. cholerae*-EFVs. From left to right: *V. cholerae* (white arrows) in vacuoles of *T. pyriformis* after overnight incubation in 0.55 × NSS at RT. The presence of many mitochondria around the vacuole is observed. The middle panel shows a magnified view of the area boxed in the first panel. The last panel focuses on a single bacterial cell in an EFV containing multiple cells to show the presence of multiple layers of membrane surrounding *V. cholerae*. Scale bars: 2 µm, 500 nm and 500 nm respectively (also see Supplementary Figure 2.). Images are representative of three independent experiments. **c**, Number of EFVs after co-incubation of *T. pyriformis* with different numbers of *V. cholerae*. Data are from four independent biological replicates (c, n=4) and shown as the mean ± SD**. d**, Numbers of EFVs produced when protein synthesis is inhibited prior to co-incubation. Data are from three independent biological replicates (d, n=3) and shown as the mean ± SD. Statistical comparisons were calculated using one-way ANOVA, Dunnett's multiple comparisons test. P**<0.01, P***<0.001.

Figure 2. Production of EFVs by different *Vibrio* spp. and ciliate wild type strains. *V. cholerae* O1 strains N16961, A1552, C6706 and HC1037 were co-incubated in 0.55 × NSS with **a**, *T. pyriformis* **b**, *T. malaccensis* **c,** *Tetrahymena* sp. and **d,** *U. marinum* at RT, and with **e,** *T. thermophila* at 30°C. **f,** Different *Vibrio* spp. incubated with *T. pyriformis*. Data

from **a-e** and **f** are from three and four independent biological replicates respectively (a-e, n=3; f, n=4) and shown as the mean ± SD. Statistical comparisons were calculated using one-way ANOVA, Tukey's multiple comparisons test. P*<0.05, P**<0.01, P***<0.001.

Figure 3. Number of EFVs produced by different *V. cholerae* mutants. Number of EFVs produced by wild type, Δ*toxR*, Δ*dacB*, Δ*ompU* and the *ompU* complemented Δ*ompU* and Δ*toxR* strains (Δ*ompU* pBAD24::*ompU* and Δ*toxR* pBAD24::*ompU*, respectively). Strains were grown in LB broth at 37ºC, 200 rpm, overnight except for the *ompU* complemented mutants that were supplemented with carbenicillin 100 μg ml^{-1} and with (for *ompU* expression) and without (control) 0.2% arabinose. Data are from three independent biological replicates (n=3) and shown as the mean ± SD. Statistical comparisons were calculated using one-way ANOVA, Dunnett's multiple comparisons test. P****<0.0001.

Figure 4. Survival of *V. cholerae* cells in EFVs under stress and starvation conditions. a, Planktonic cells (open bars) and *V. cholerae*-EFVs (closed bars) were incubated for 60 min in 0.55 × NSS adjusted to pH 3.4. The number of colony forming units (CFU) were determined every 20 min by treating the samples with 1% Triton X-100 (Supplementary Fig. 4a-c), serial dilution and plating on LB agar plates. Data are from three independent biological replicates (n=3) and shown as the mean ± SD. **b**, *V. cholerae*-EFVs and planktonic cells were treated (closed bars) or not (open bars) with gentamicin (300 μg ml^{-1}) for 1 h at RT and the number of colonies determined. Data are from three independent biological replicates (n=3) and shown as the mean ± SD. Statistical comparisons were calculated using two-way ANOVA, Sidak's multiple comparisons test. NS= not significant, P****<0.0001. **c**, CFUs of cells within EFVs (red) and planktonic cells (blue) incubated in 0.55 × NSS for 12 weeks. Data are from three independent biological replicates (n=3) and shown as the mean ± 95% confidence intervals.

Figure 5. Escape of *V. cholerae* from EFVs under different nutrient and temperature conditions. **a**, Escape of *V. cholerae* from EFVs. *V. cholerae*-EFVs were incubated in LB broth at 37ºC and the EFVs time-lapse imaged for 3 h. The upper left picture shows a single *V. cholerae*-EFV (white arrow) suspended in LB broth at the beginning of the incubation. The upper right picture shows the rupture of the EFV membrane after ~10 min (white arrow) with the subsequent release of *V. cholerae* cells (black arrow). The bottom left shows *V. cholerae* cells actively dividing and escaping from EFVs (white arrow) with more extracellular bacteria present (black arrow). The bottom right shows dense growth from EFVs (white arrow) and many extracellular *V. cholerae* cells show active division (black arrow). **b**, Incubation of EFVs at 37ºC without carbon or nutrient source (suspended in 0.55 × NSS). White arrows show two EFVs at time 0. A single EFV (white arrow) showing many *V. cholerae* cells after 4 h of incubation. Single EFV (white arrow) and extracellular bacteria (black arrows) are observed after 24 h of incubation. **c**, EFVs incubated in LB broth at RT. The video started recording after 2 h of incubation. The first image shows a single *V. cholerae*-EFV. The second image shows rupture of the EFV membrane (white arrow) and the third, *V. cholerae* cells showing active growth from the EFV (white arrow). **d**, EFVs (white arrows) suspended in 0.55 × NSS at RT for 2 months. Intact EFVs are observed without extracellular bacteria. Scale bars from **a-d** are 10 µm. Images (a-d) are representative of three independent experiments.

Figure 6. CI for *in vitro* and *in vivo* assays of *V. cholerae*-EFVs versus planktonic *V. cholerae* and working model. **a**, CI of *in vitro* and **b**, *in vivo* assays calculated by the output ratio after incubation (*in vitro*: overnight, 37°C; *in vivo*: 24 h, 24°C) corrected by the input ratio. CFUs were assessed by plating on LB10 agar plates supplemented with rifampicin 100 µg ml^{-1} to inhibit other intestinal bacteria and X-gal 80 µg ml^{-1}. Data from **a** and **b** are from sixteen independent biological replicates (n=16) and shown as the median. The CI of *V.*

cholerae-EFVs compared to planktonic cells *in vitro* and *in vivo* assays is significantly higher than an hypothetical median of 1.0 (Two-tailed, non-parametric, Wilcoxon Signed Rank Test. P****<0.0001). **c**, CI of *in vitro* and **d**, *in vivo* assays performed with competitions of the 6-week-old EFVs (incubated in 0.55 × NSS at RT) and 6-week-old planktonic cells (control, incubated in 0.55 × NSS at RT) against the Δ*lacZ* wild type was calculated by the output ratio after incubation (*in vitro*: overnight, 37°C; *in vivo*: 24 h, 24°C) corrected by the input ratio. CFUs were assessed by plating on LB10 agar plates supplemented with rifampicin 100 μg ml^{-1} to inhibit other intestinal bacteria and X-gal 80 μg ml^{-1}. Data from **c** and **d** are from five independent biological replicates (n=5) and shown as the median. The CI of the 6-week-old *V. cholerae*-EFVs compared to the 6-week-old planktonic cells (control) was significantly different in both *in vitro* and *in vivo* conditions (Two-tailed, non-parametric, Mann-Whitney Test. P**<0.01). **e**, *V. cholerae*-EFVs incubated at 37°C for 4 h in 0.55 × NSS at pH 3.4 shows intact *V. cholerae*-EFVs untreated (left panel) and treated (right panel) with deoxycholic acid (0.4 %). The white arrows show digested EFVs after deoxycholic acid treatment. Scale bars are 10 μm. Images are representative of three independent experiments.

Table 1. Numbers of EFVs produced by different *V. cholerae* mutants.

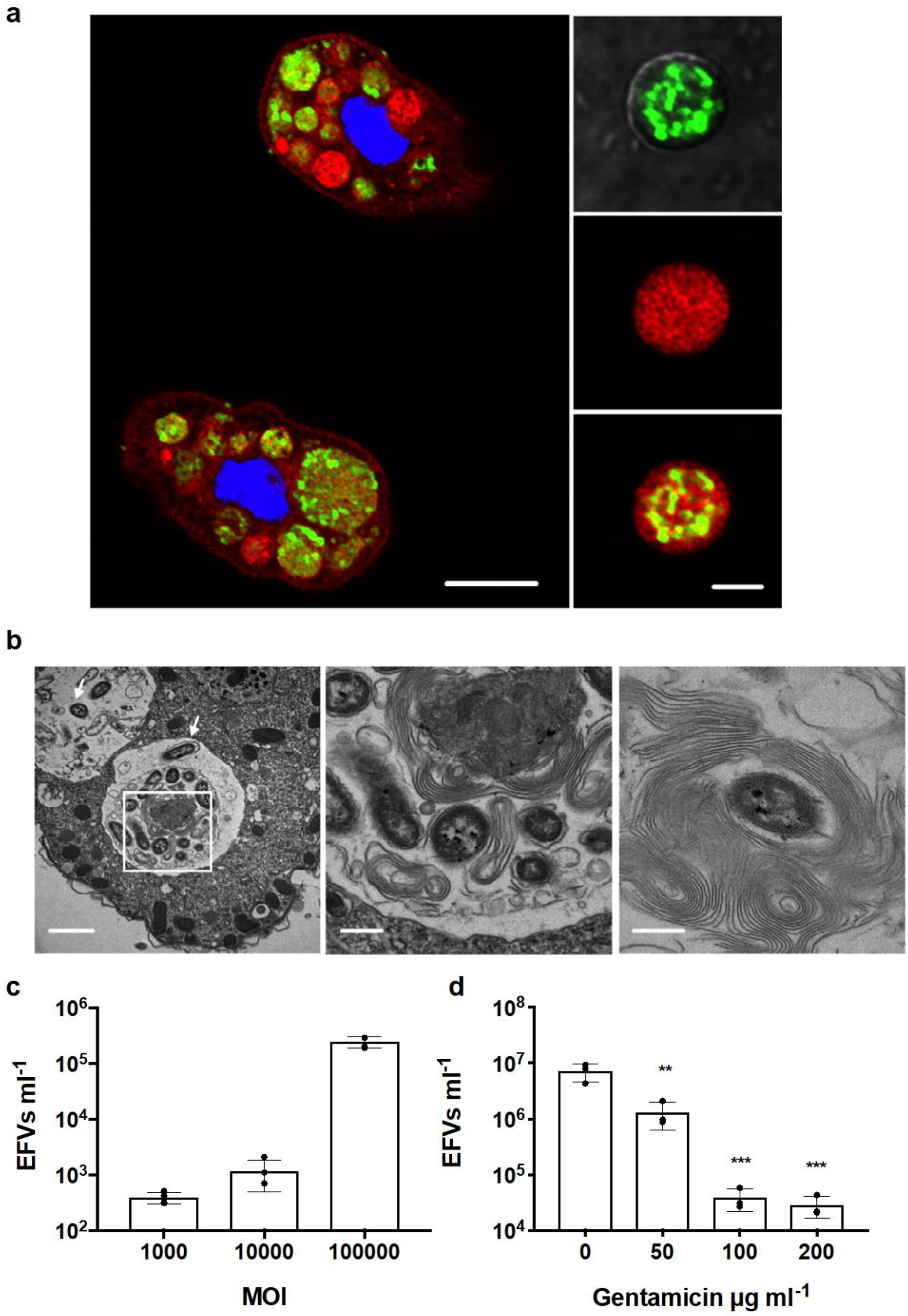
Classification	**A1552* Mutants Tested**	**Number of EFVs ml^{-1}** (Mean ± SD)
Grazing resistance/Biofilm formation	Δ*vpsA*	4.15E+05 ± 6.81E+04^{ns}
	Δ*rpoS*	3.93E+05 ± 1.30E+05^{ns}
	Δ*hmgA*	3.78E+05 ± 1.34E+05^{ns}
	Δ*vpsR*	2.73E+05 ± 1.30E+05^{ns}
Transcriptional regulators	Δ*hapR*	4.50E+05 ± 1.44E+05^{ns}
	Δ*phoB*	2.75E+05 ± 1.02E+05^{ns}
	Δ*chiS*	5.40E+05 ± 9.76E+04^{ns}
	Δ*toxR*	1.28E+04 ± 3.95E+03^{s}
Motility	Δ*flaA*	3.20E+05 ± 1.83E+05^{ns}
Acid resistance	Δ*cadC*	3.50E+05 ± 1.96E+05^{ns}
Outer membrane proteins	Δ*ompU*	4.25E+03 ± 5.00E+02^{s}
	Δ*ompV*	4.03E+05 ± 1.44E+05^{ns}
Aminoacyl lipid modification	Δ*almEFG*	2.97E+05 ± 1.15E+05^{ns}
Type I secretion system	Δ*rtxA*	4.00E+05 ± 1.67E+05^{ns}
Type II secretion system	Δ*CTXΦ*	2.85E+05 ± 1.98E+05^{ns}
	Δ*gbpA*	3.90E+05 ± 1.95E+05^{ns}
	Δ*lapA*	3.20E+05 ± 1.68E+05^{ns}
Type VI secretion system	Δ*hcp1*	3.85E+05 ± 1.55E+05^{ns}
	Δ*hcp2*	3.65E+05 ± 6.19E+04^{ns}
	Δ*hcp1,2*	3.80E+05 ± 1.76E+05^{ns}
Intracellular survival and multiplication in other bacteria	Δ*ankB*	2.98E+05 ± 2.08E+05^{ns}

650 *Sample size n=4;
ns: No significant reduction compared to WT;
652 s : Significant reduction compared to WT.

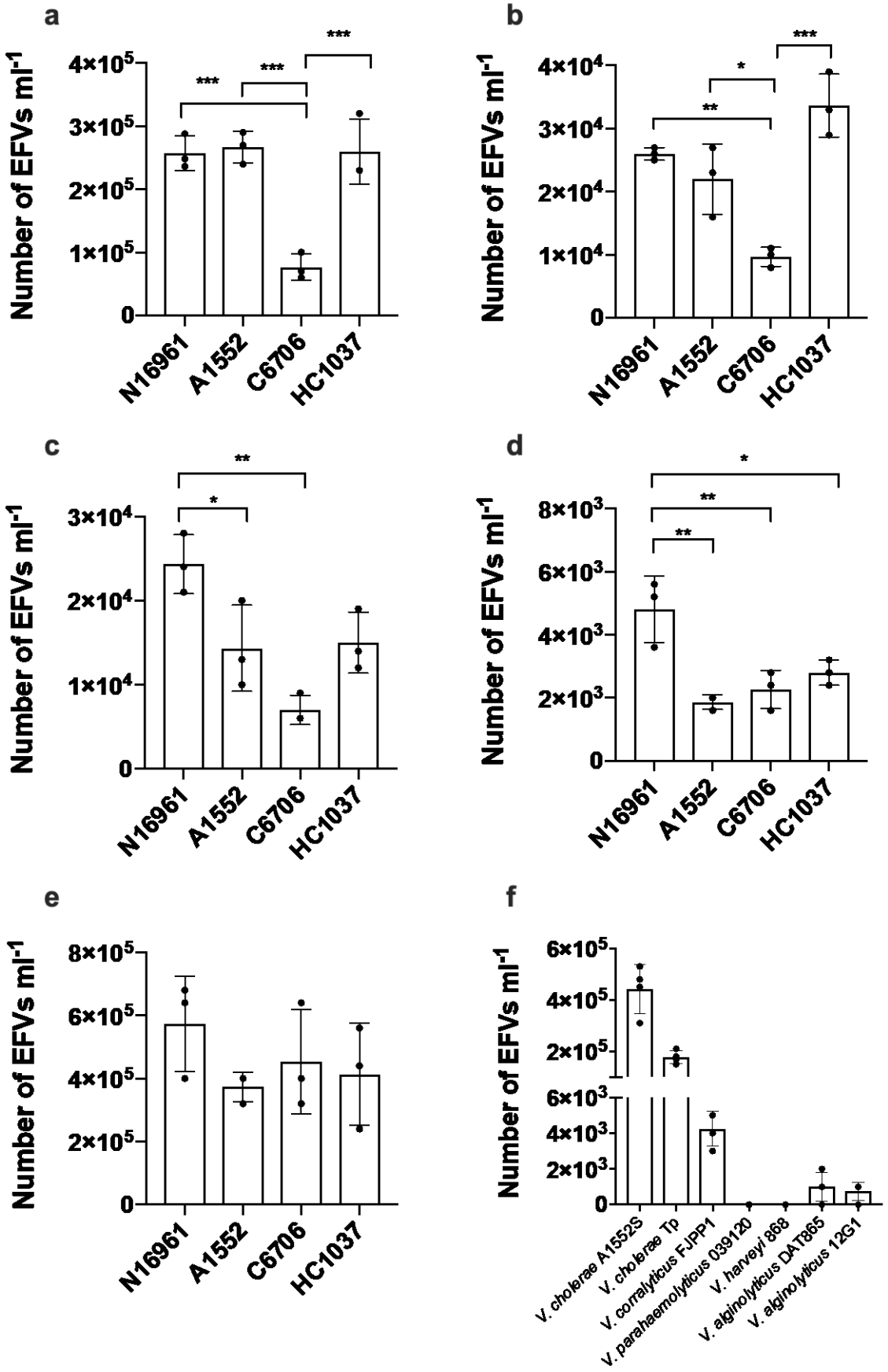
654

Figures

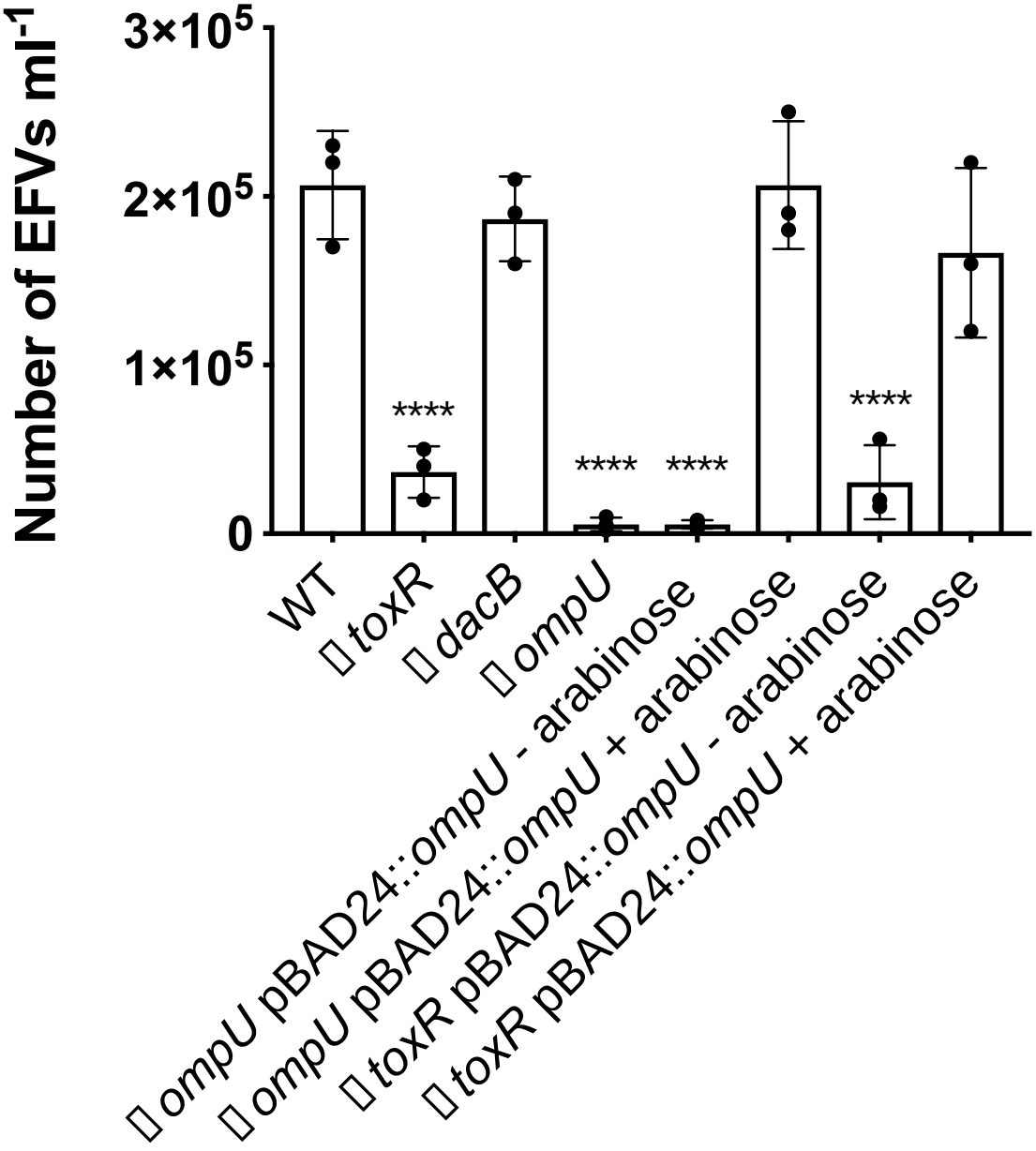
Figure 1.



658 Figure 2.

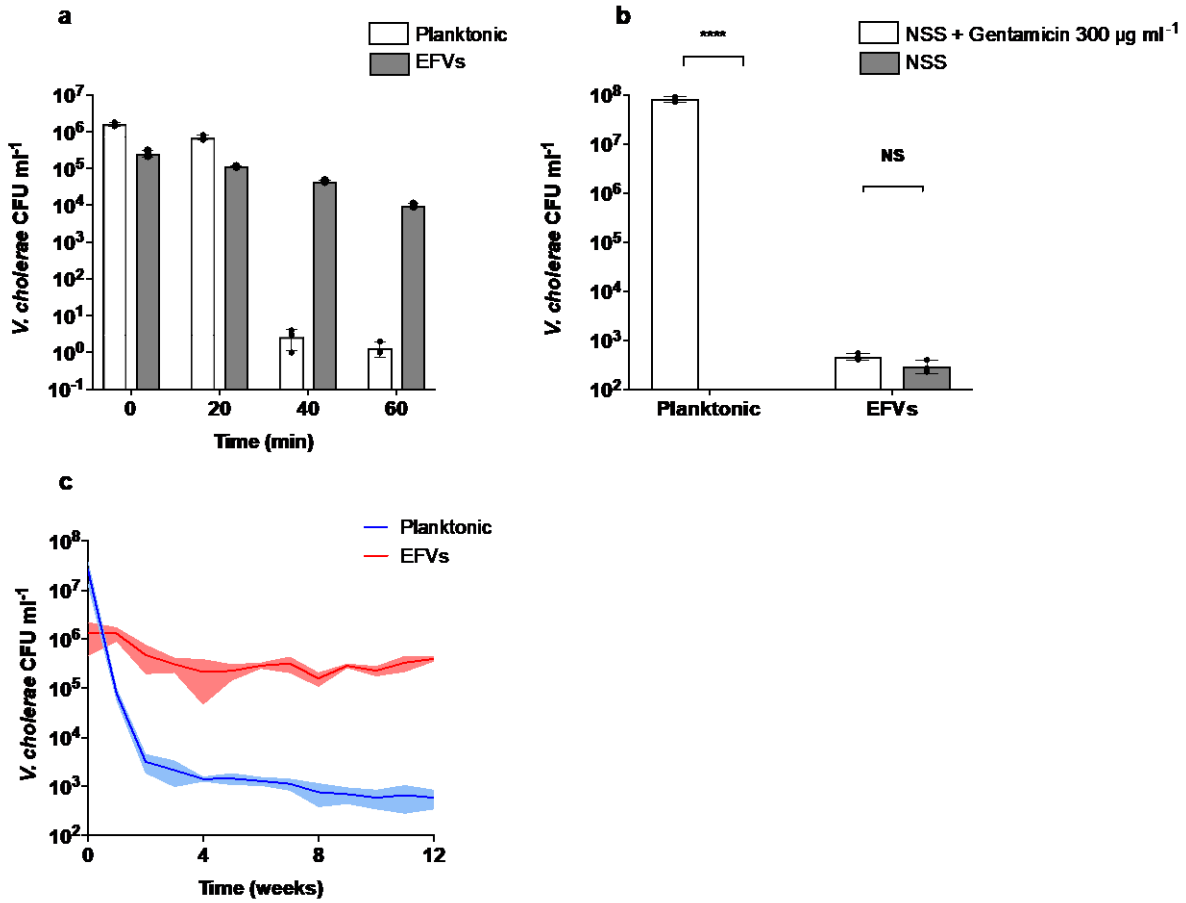

a
b
c
d
e
f
Number of EFVs ml-1
4×10^5
3×10^5
2×10^5
1×10^5
0
4×10^4
3×10^4
2×10^4
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
6×10^5
4×10^5
2×10^5
6×10^3
4×10^3
2×10^3
***
**
*
N16961
A1552
C6706
HC1037
V. cholerae A1552S
V. cholerae Tp
V. coralyticus FJPP1
V. parahaemolyticus 039120
V. harveyi 868
V. alginolyticus DAT865
V. alginolyticus 12G1

660 Figure 3.



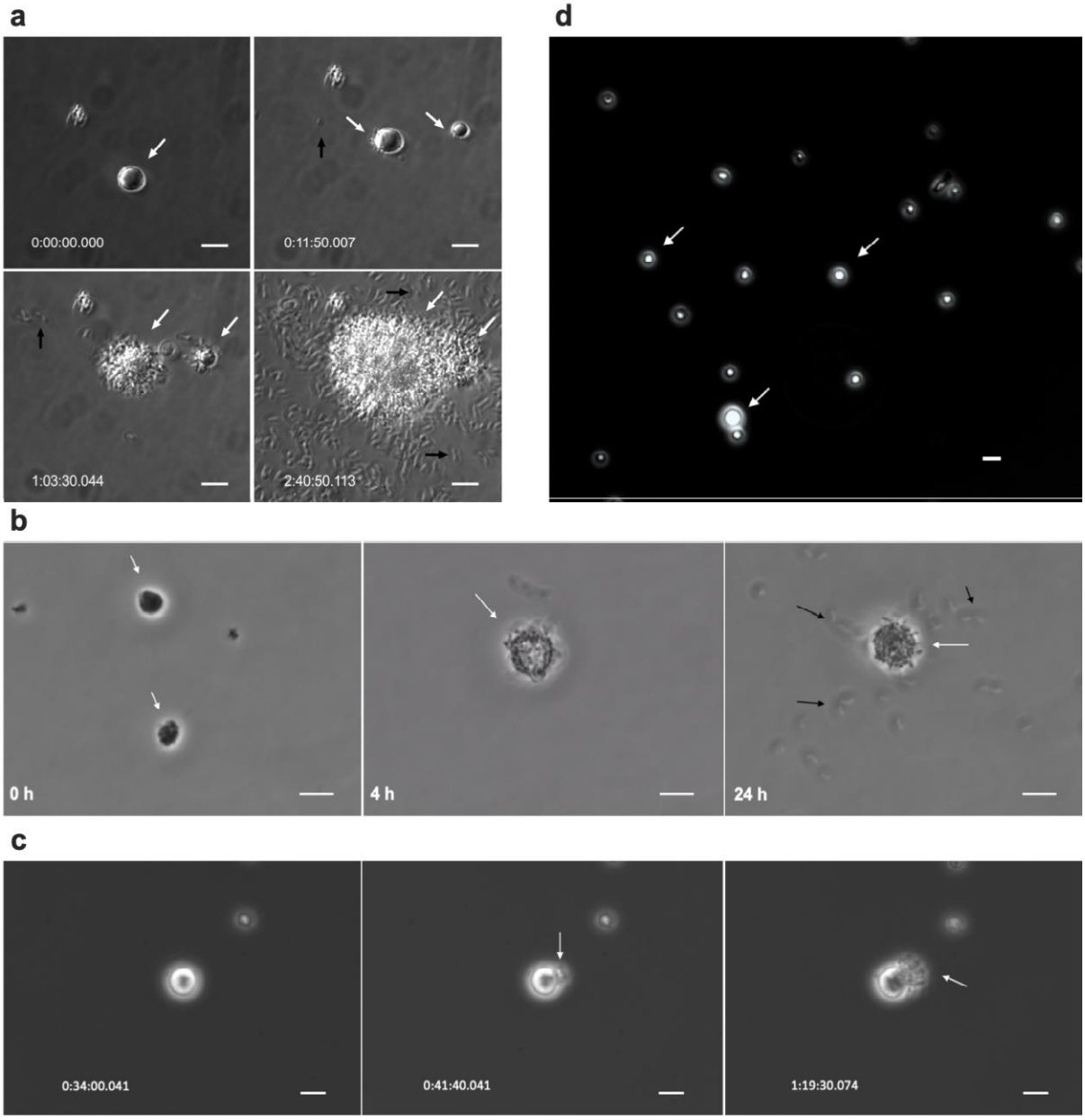
662

Figure 4.


a
Planktonic
EFVs
V. cholerae CFU ml-1
Time (min)
b
NSS + Gentamicin 300 µg ml-1
NSS
****
NS
Planktonic
EFVs
c
Planktonic
EFVs
V. cholerae CFU ml-1
Time (weeks)

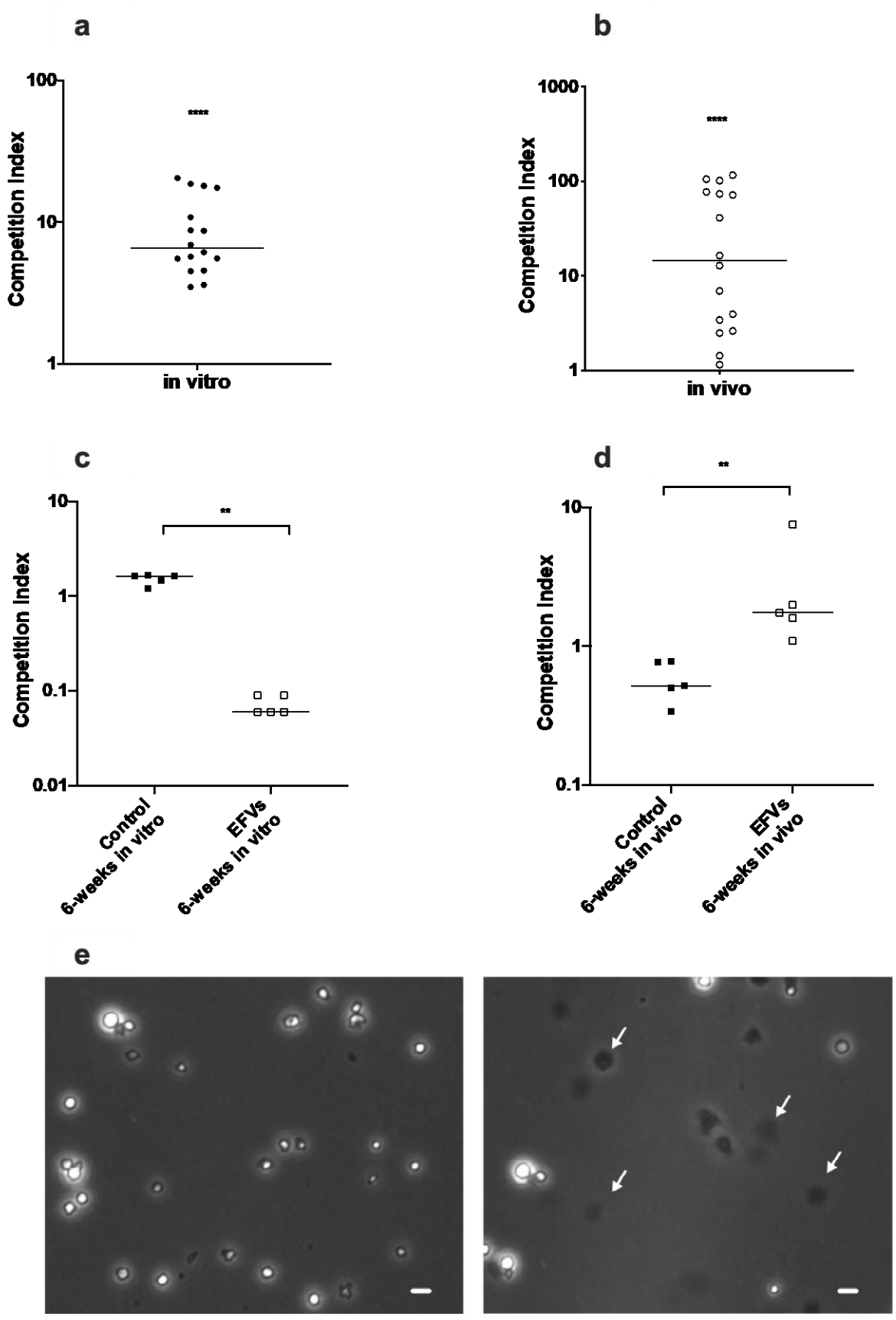
664

666 Figure 5



668

Figure 6.


a
100
10
1
Competition Index
****
in vitro
b
1000
100
10
1
Competition Index
****
in vivo
c
10
1
0.1
0.01
Competition Index
**
Control
6-weeks in vitro
EFVs
6-weeks in vitro
d
10
1
0.1
Competition Index
**
Control
6-weeks in vivo
EFVs
6-weeks in vivo
e

670